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(54) Title: <b>METHOD AND DEVICE FOR IDENTIFYING A MYCOBACTERIUM SPECIES RESPONSIBLE FOR A MYCOBACTERIAL INFECTION</b>		
(57) Abstract  <p>The invention relates to a method for identifying a <i>Mycobacterium</i> species responsible for a mycobacterial infection in human or animal, comprising selecting a suitable mycobacterial species and strain; preparing at least one mycobacterial antigen, respectively antigen preparation; binding the antigen, respectively the antigen preparation to a suitable carrier; causing the binding antigen to react with antibodies from serum of an individual infected with a <i>Mycobacterium</i> species; making visible antigen-antibody reactions for a suitable antibody (sub-)class; and identifying the responsible <i>Mycobacterium</i> species on the basis of the reactions which are made visible. The invention further provides a diagnostic kit which takes the form of a dip-stick on which is arranged a carrier strip with mycobacterial antigens binding thereto, and means for visualizing antigen-antibody reactions occurring on the carrier after contact with the serum for testing. In another embodiment the diagnostic kit comprises a microtiter plate, in the wells of which a specified antibody is arranged, and means for making visible antigen-antibody reactions occurring in the wells after contact with the serum for testing. The third embodiment is an immunoblot with mycobacterial antigens separated by electrophoresis binding thereto, and means for visualizing antigen-antibody reactions occurring on the immunoblot after contact with the serum for testing.</p>		

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## METHOD AND DEVICE FOR IDENTIFYING A MYCOBACTERIUM SPECIES RESPONSIBLE FOR A MYCOBACTERIAL INFECTION

The invention relates to a method for identifying a Mycobacterium species responsible for a mycobacterial infection in human or animal. The invention further relates to diagnostic kits for use in the method.

5       The genus Mycobacterium which contains about 50 species is responsible for a number of human and animal diseases which are known collectively as the mycobacterioses. The best known of these in humans is leprosy, caused by M. leprae, which affects more than ten million people  
10 worldwide, and tuberculosis, usually caused by M. tuberculosis, at least ten million new cases of which occur each year. Most other mycobacteria normally occur only as environmental saprophytes but can also cause opportunist diseases. This happens usually, but not only, in the case of  
15 people who have problems with their immune system, such as AIDS patients and people undergoing immunosuppression. These opportunist types comprise the slow-growing species M. avium, and the closely related M. intracellulare and M. scrofulaceum (often referred to together as MAIS complex),  
20 M. kansasii, M. marinum and M. ulcerans, and the fast-growing species M. chelonae and M. fortuitum. Although once rare, the incidence of opportunist mycobacterial diseases and tuberculosis shows a parallel increase in the western world with the incidence of AIDS. In addition there is limited but  
25 increasing evidence that mycobacteria or antigens thereof play a direct or indirect part in the etiology of a plurality of other diseases such as sarcoidosis and Crohn's disease and different auto-immune diseases such as auto-immune dermatitis, rheumatoid arthritis and diabetes. This  
30 could be attributed to a structural mimicry between epitopes of mycobacteria and those of the host.

The cell walls of mycobacteria are very complex and contain many lipids, some with structures unique to the

genus. These structures comprise mycolinic acids and esters, peptido-glycolipide, arabino-galactane and lipo-arabino-manane. The lipid-rich mycobacterial cell walls are responsible for the characterizing colouring properties of the mycobacteria. They also enable mycobacteria to counter an attack by the immune system of the host. A number of species, once taken up into macrophages, are capable of surrounding themselves with a thick layer of secreted lipids.

10 Many different components of the mycobacteria begin an interaction with the immune system. These components comprise protein and hydrocarbon antigens, which can either be actively secreted by the mycobacteria or can form part of the cell wall or cell membrane. In addition they may be  
15 present in the cytoplasm, for instance in the cytoplasmic matrix, ribosomes and enzymes. Mycobacteria also possess immuno-modulating components such as immunosuppressing compounds and adjuvants. Consequently, a single mycobacterial species can induce a large variety of immune  
20 responses in different forms and with diverse specificities. It is therefore difficult to distinguish immune responses against species-specific components from cross reactions. For this reason it has therefore been found difficult to derive protein antigens suitable for the detection of  
25 species-specific humoral responses as a basis for a very sensitive and specific sero-diagnostic test for tuberculosis. Because the mycobacteria occur a great deal in the environment, human serum nearly always contains anti-mycobacterial antibodies.

30 In view of the problems with the specificity of protein antigens, a number of researchers, including the present inventors, have focused their attention on species-specific glycolipid antigens for the detection of specific humoral immune responses. Although the immune reactivity against  
35 mycobacteria is of the cell-mediated type and the humoral immune responses probably play a minor part in the total effector mechanism of mycobacterial immunity and immunopathology, studies into the antibody response to

immuno-dominant mycobacterial cross-reactive antigen components (further referred to as Im-CRAC) could shed light on the varying capability of the host to recognize different mycobacterial antigens. They could therefore provide  
5 indirect information relating to the nature of the immune recognition of, and response to, a specific mycobacterial pathogen.

It has now been found that the clinical manifestation of mycobacterial diseases appears to be related to the  
10 varying capability of an individual host to produce a humoral response to different mycobacterial immuno-cross-reactive antigen components (Im-CRAC). Each mycobacterial infection generates its own specific antibody response to a number of specified antigens. Analysis of the antibody-  
15 response by means of immunoblotting has demonstrated that the immuno-dominant Im-CRAC vary in accordance with the immunopathological manifestation of the mycobacterial diseases. It has been found that the sera of individuals which are infected with different Mycobacterium species  
20 cause different and distinguishing band patterns on immunoblots of mycobacterial antigens.

This discovery forms the basis of the present invention, whereby a method is provided for identifying a Mycobacterium species responsible for a mycobacterial  
25 infection in human or animal, comprising the steps of:

- a) selecting a suitable mycobacterial species and strain;
- b) preparing at least one mycobacterial antigen, respectively antigen preparation;
- 30 c) binding the antigen, respectively the antigen preparation to a suitable carrier;
- d) causing the binding antigen to react with antibodies from serum of an individual infected with a Mycobacterium species;
- 35 e) making visible antigen-antibody reactions for a suitable antibody (sub-)class; and
- f) identifying the responsible Mycobacterium species on the basis of the reactions which are made visible.

In preference the antigen preparation is separated by electrophoresis prior to step c) and the carrier is a membrane to which the antigen is bound by means of electroblotting. This process is called Western blotting.

5       The Im-CRAC comprise namely a number of antigens with specific molecular weights which, as has now been found, after immunoblotting exhibit a binding pattern which correlates to the disease, respectively infection. The specific band pattern is characterized by the presence or  
10 absence of four individual components, for instance:

- a region comprising different pronounced bands and/ or overlapping bands, which can be observed as a smear ("region");
- sharp single bands which are strongly positive  
15 ("band");
- sharp double bands which are strongly positive ("doublet"); and
- other positive bands ("extra bands").

For a survey of the different antigens, their molecular  
20 weights and binding characteristics, see table 1.

Table 1:

Survey of characteristic binding patterns of mycobacterial  
Immuno-Cross-reactive Antigen Components.

5

10

15

Antigen	Diagnostic for	MW range (in KDa)	Binding characteristic
A	J	< 8	band
B	T, B, J,	10-16	band
C	B, J, T,	20-28	band or region
D	L	29-33	doublet
E	B, J, T,	31-40	band or region
F	T	38-40	band or region
G	C, B, J,	45-48	doublet
H	T	58-60	band or region
I	L	64-65	doublet
J	J	66	band
K	T	68	band
L	L	30-64	region

20

T: Human Tuberculosis  
L: Leprosy  
C: Crohn's Disease  
B: Bovine Tuberculosis  
J: Johne's Disease

25

The mycobacterioses are all characterized by a specific  
banding pattern which is formed when a blot having thereon  
an antigen preparation of mycobacteria separated to size is  
incubated with serum of an infected individual.

30

The tables 2a-2e below show a survey of the banding  
patterns of a number of mycobacterial, respectively  
immunological diseases.

Table 2a:

Bovine tuberculosis

Regions (MW in KDa)

5	Pattern	and/or 10-16	and/or 20-28	and/or 31-40	and 45-48
	1.	14 KDa band	22 KDa band 20-28 KDa region	31 KDa band	45/48 KDa doublet

John's Disease

Regions (MW in KDa)

10	Pattern	and/or > 8	and 10-16	and/or 20-28	and/or 31-40	and 45-48	and 66
	1.	region	14 KDa band	and 22 (25)- KDa band, and/or 27 KDa band	31 KDa band	45/48 KDa doublet	66 KDa band



Table 2b:

## Human Tuberculosis

Regions (MW in KDa)

	Pattern	10-16	20-28	31-38	38-40	58-60	68	other*
5	1.	10 and 16 KDa bands	/	33 KDa band	region	bands	/	+/-
10	2.	16 KDa single band	region	33 KDa band	bands	region	/	+/-
	3.	10 or 16 KDa band	/	/	bands	bands	/	+/-
15	4.	16 KDa band	/	33 KDa band	region	bands	/	+/-
20	5.	10 and/or 16 KDa band	region	33 KDa band	bands	bands and/or region	68 KDa band	+/-

\* Extra bands and/or regions can colour but are not diagnostic for Human Tuberculosis

Table 2c:

## Leprosy

## Regions (MW in KDa)

5	Pattern	29-33	30-65	64-65	other*
	LL pattern 1	29/33 KDa doublet	/	/	+/-
	TT pattern 1	/	regions and/or bands	/	+/-
10	TT pattern 2	/	regions and/or bands	64/65 KDa doublet	+/-
15	TT pattern 3	/	/	64/65 KDa doublet	+/-

\* Extra bands and/or regions can colour but are not diagnostic for leprosy

20

Table 2d:

## Crohn's Disease

## Regions (MW in KDa)

25	Pattern	45-48	other*
	1.	45/48 KDa doublet	+/-

\* Extra bands and/or regions can colour but are not diagnostic for Crohn's Disease

Table 2a:

5

Regions (MW in KDa)

Pattern	and 42 KDa	and/or 80-90 KDa	and/or 58-60 KDa	and/or 14-18 KDa
1.	band	region	region	region

When an immunoblot is used, two questions can be  
 10 answered. Firstly, the presence of any positive band pattern  
 will answer the question of whether a mycobacterial  
 infection is present. Secondly, the presence of specific  
 banding patterns indicates which mycobacterial species has  
 caused the infection, and therefore what the nature and  
 15 etiology of the disease will be.

The invention further relates to a heterogeneous enzyme  
 immunoassay.

From the patterns in the immunoblotting it follows  
 which mycobacterial antigens respectively antigen prepara-  
 20 tions are suitable for diagnosis of any particular disease.

The antigen for a heterogeneous enzyme immunoassay is  
 preferably chosen from the group which consists of myco-  
 bacterial immuno-cross-reactive antigen components with a  
 molecular weight of 29-33 KDa, 45-48 KDa, 64-65 KDa and a  
 25 fraction designated with the term KP-100. These ImCRAC can  
 be used separately or in combination with each other for  
 serological diagnosis of the correlating diseases in a  
 heterogeneous enzyme immunoassay (EIA).

In this form of assay antibody-conjugates labelled with  
 30 a standard enzyme are used. An important detail is that the  
 enzyme activity does not change during the immunological  
 reaction.

In order to test the immune response in patients to the  
 selected antigens, use is made for instance of microtiter  
 35 plates ("Solid Phase"). By means of standard published

techniques the antigens are irreversibly immobilized on the surface of the wells in such a microtiter plate.

This binding takes place while retaining specific antigen determinants on the used antigens. After incubation  
5 with serum, in the wells of the microtiter plate, antibodies present therein can specifically form a complex with the irreversibly bound antigens.

After removal of non-binding serum components, binding antibodies are detected using an anti-antibody antibody  
10 labelled with an enzyme.

Binding of the enzyme is only possible when specific antibodies have adhered to the immobilized antigens. Substrate conversion by the binding enzyme to a visually or photometrically observable signal is thereby directly  
15 related to the presence of specific antibodies in the tested serum.

The choice of specificity of the enzyme-bound anti-antibody antibody determines the type of reaction that takes place. For instance, it may be desirable in some cases to  
20 demonstrate the specifically binding immunoglobulins of the IgG type, while in other cases immunoglobulins of the IgA and/or IgM type are just demonstrated.

The combination of antigen and immunoglobulin type defines the specificity of the test.

25 The said methods, that is, the immunoblot and the EIA, can be used as mutual confirmation.

In addition, for the serological diagnosis based on the said antigens, use can be made of a test stick as solid phase.

30 A particularly advantageous embodiment of the invention relates to a test stick, the so-called "dip-stick", which is used as solid phase in the heterogeneous enzyme immunoassay.

The said mycobacterial antigens can be irreversibly bound to such a dip-stick.

35 The antigen is brought into reaction with antibody from serum for testing by dipping the dip-stick in a serum sample for testing. The formed antigen-antibody complex can be made

visible by subsequently dipping the dip-stick in an anti-antibody antibody-enzyme conjugate solution.

With the binding enzyme a substrate can then be converted to a visually or photometrically observable signal.

The invention provides a diagnostic kit for:

- an immunoblot assay; comprising IMCRAC antigens separated by electrophoresis as described above, immobilized on a solid carrier, in addition to an associated suitable detection system.

- a heterogeneous enzyme immunological assay; comprising a microtiter plate, the wells of which are coated with above mentioned antigens or antigen preparations, in addition to an associated suitable detection system.

- a dip-stick assay; comprising test sticks coated with antigen or antigen preparations, in addition to an associated suitable detection system.

The present invention will be further elucidated with reference to a number of examples which are given herein by way of illustration and are not intended to limit the invention.

#### EXAMPLE 1 Immunoblot

1. Preparation of crude mycobacterial mass ("starting material").

The mycobacteria were cultured in commercially available Sauton medium supplemented with 2 g  $\text{MgSO}_4$ , 8 g citric acid, 2g  $\text{K}_2\text{HPO}_4$ , 16 g asparagine, 2 g  $(\text{Fe}^{3+})$  ammonium citrate, 240 ml glycerol. The bacteria were cultured under standard conditions. The cells were harvested by filtration of the culture medium with a 12  $\mu\text{m}$  filter. The cells were subsequently resuspended in 20 ml PBS (phosphate-buffered salt solution) (pH 7.4) and the harvested cells were autoclaved under a pressure of 15 Psi for 20 minutes in order to deactivate and sterilize the bacteria. The thus obtained bacterial mass can be stored at  $-80^\circ\text{C}$ .

In order to determine the quantity of starting material a 1/100 dilution of the harvested autoclaved suspension in

PBS was made. The optical density thereof, measured at 420 nm (O.D.<sub>420</sub>) must be 0.1. If necessary the concentrated bacterial mass is supplemented with PBS (pH 7.4) until the correct O.D. is obtained. An O.D.<sub>420</sub> of 0.1 indicates the presence of  $7 \times 10^8$  bacteria per 30 ml, which is equivalent to 12 g wet weight of the bacterial mass.

For preparation of a crude mycobacterial extract 5 g wet weight of the bacterial mass was washed three times with PBS (pH 7.4). Centrifuging was then carried out at 3000 x g until the mass precipitated. The pellet was suspended in 50 ml PBS and stirred carefully to reduce formation of lumps to a minimum. To prevent lump forming 0.05% Tween 80 was optionally added. In order to avoid bacterial contamination 3 mg penicillin/streptomycin was added to this solution. The concentration was then brought with PBS to 2 g wet weight/ml.

The bacterial mass was subsequently broken open using an automatic French-X-press or RIBI press (American Instruments Company, Trevenollab. Inc. Maryland). The buckets were pre-cooled overnight at -20°C. Before use the buckets were held in a mixture of ethanol and dry ice (-20°C). After the buckets were filled with 1 g bacterial mass per bucket of 5 ml and cooled at -80°C for 20 minutes, the buckets were placed in the French-X press and 12 tons of pressure were applied by pushing in the plunger of the press. The buckets were then removed and cooled again at -80°C for 20 minutes. The buckets were inverted and treated for the second time. 10 tons of pressure were applied the second time. Cooling and breaking were then repeated a number of times, normally about 5 times. The disrupted cells were eluted with a suitable volume of PBS and subsequently centrifuged at 4°C at 300 x g for 10 minutes in order to remove the unbroken bacteria with the sediment. The collected supernatant was then centrifuged at 4°C and 145,000 x g for 2 hours. The pellet was suspended in 0.1 M Tris-HCl (pH 7.2), 0.01 M EDTA which contained 20 mM MgSO<sub>4</sub>·7H<sub>2</sub>O in a concentration of about 1 g per 10 ml. 1 mg RNase and 1 mg DNase were added per 10 ml volume. Incubation then took place overnight at 4°C with

careful stirring. Incubation thereafter took place for 1 hour at 37°C and the lysate was centrifuged at 300 x g and 4°C for 10 minutes in order to remove the last-remaining unbroken bacteria (this is further referred to as "starting material").

## 2. Manufacture of membrane for assays.

A 12% polyacrylamide analytical gel of 1.5 mm thickness was manufactured according to normal standard procedures. No comb was used in the stacking gel. 5 mg of the starting material obtained under 1. (100 mg per ml), respectively KP-100 or SP-100 (see example 2) were used for each gel. 40 microlitres of this material was diluted with 1200 microlitres PBS. 300 microlitres 5x loading mixture (0.3 g 15 250 mM Tris-HCl, 1.0 g 10% SDS, 1.0 g 10% dithioerytreitol, 5 mg 0.05% bromophenol blue) was then added.

Incubation was carried out for 20 minutes at 65°C. 1500 microlitres were subsequently applied to the gel and electrophoresis performed under the following conditions: 20 150 V for the run through the stacking gel for 30 minutes and 100 V through the running gel for 6 hours.

In order to form a Western blot the proteins present in the gel were transferred at 50 V for 3 hours to a nitrocellulose membrane. After completion of the transfer 25 the membrane was coloured with 1.5% amido black for 2 minutes to check the membrane for irregularities and air bubbles. The membrane was then decolourized in 0.05% Tween 80 in PBS with 1% BSA (bovine serum albumin). The membrane was then cut into strips and was ready for use.

30

## 3. Immunodetection

The strips were incubated with 1:200 diluted human serum. The serum was diluted in PBS with 3% BSA. The incubation took place for 1 hour at room temperature. The 35 strips were subsequently washed three times (for 3 minutes at a time) in PBS. The strips were then incubated with a goat anti-total-human immunoglobulin-alkaline phosphatase conjugate in a dilution of 1 to 1000 in PBS with 3% BSA and

0.05% Tween 80. Washing in PBS then took place again three times. The colour was developed with an NBT/BCIP (nitroblue tetrazolium/Bromo, Chloro Indolyl phosphate) colour solution (1 mg per 10 ml) to which 10 microlitres  $H_2O_2$  were added.

5 The strips were incubated for a maximum of 2 hours in 1 ml of this solution per strip. The colour reaction was stopped by transferring the strips to 0.1 M Tris-HCl (pH 8.3), 0.01 M EDTA. The obtained patterns are interpreted by comparison with a reference pattern.

10 The results are shown in figures 1 and 2a-2c.

Figure 1 shows in the blots A and B an example of Western blotting patterns which are developed after incubation respectively with representative negative and positive sera (positive for bovine tuberculosis).

15 Blots C and D are an example of Western blotting patterns which are developed after incubation with a representative negative and positive serum sample, respectively (positive for Cattle Jones Disease).

20 Blots A and B: Lane 1: BCG crude extract, Lane 2: crude extract of an M. tuberculosis strain, Lane 3: Myc. bovis crude extract.

Blots C and D: Lane 1: BCG derived KP-100, Lane 2: RIVM 7114 derived KP-100.

25 Interpretation of banding patterns from left to right is as follows.

Only the specific characteristics are stated herein.

Blot A: only the background bands can be observed in blots incubated with negative serum.

30 Blot B: region in the 10-16 KDa region in lane 3, 22 KDa band in lane 2, 31 KDa bands in lane 1 and 2, 14 KDa band in lane 2.

Blot C: only background bands can be observed in blots incubated with negative serum.

35 Blot D: 45-48 KDa doublet in lane 1 and 2, 22 and 25 KDa band in lane 1 and 2, 66 KDa band in lane 1 and 2, 27 KDa band in lane 1.



Figure 2a is an example of different Western blotting patterns developed after incubation with representative variable sera of tuberculosis patients. Noticeable is the combination of different patterns demonstrating the presence of different dominant bands, as shown in table 1. These band patterns function as "hallmarks" for TB patients as diagnosed serologically.

Applicable to all blots (from left to right): Lane 1 = BCG crude extract, Lane 2 = crude extract of an M. tuberculosis strain.

Interpretation of banding patterns is as follows. Different blots (originating from different PAGE gels) are herein compared with each other.

Blot A: Mycobacterium avium infected patient.

Blot B-F: Tuberculosis patients.

Blot G: non-endemic negative serum.

Blot H: endemic negative serum (known recent contact, blot developed 2 weeks after patient returned to Netherlands from endemic range).

Only "hallmarks" are mentioned.

Blot A: Mycobacterium avium infected patients, sera, band at 68 KDa in lane 1 and 2, range in 10-16 KDa in lane 1, band in the 58-60 KDa region in lane 2. Patient shows low IgA titer in P-90 ELISA).

Blot B: 38-40 KDa band in lane 1 and 2, 10-16 KDa band in lane 1 and 2, band in 58-60 KDa region in lane 2, smear in 22-28 KDa region in lane 1.

Blot C: 16 KDa band in lane 1 and 2, bands in 58-60\* KDa region in lane 1 and 2, bands in 38-40 KDa region in lane 1 and 2, smear in 22-28 KDa region in lane 1, 33 KDa band in lane 1 and 2.

Blot D: 10 KDa band in 10-16 KDa region in lane 1, 16 KDa band in 10-16 KDa region in lane 2, 68 KDa band in lane 1 and 2, bands in 58-60\* KDa region in lane 1 and 2.

Blot E: smear in 33-38 KDa region in lane 1 and 2, 16 KDa bands in lane 1 and 2, bands in 58-60\* KDa region in lane 2.

Blot F: bands in 10-16, 22-28, 38-40, 58-60 regions and 68 KDa band in both lanes 1 and 2.

Blot G: non-endemic negative serum.

Blot H: endemic negative serum (known contact).

5        Figure 2b is an example of different Western blotting patterns developed after incubation with representative sera of patients with Lepromatous Leprosy (LL), Blot A and C, and Tuberculous Leprosy (TT), Blot B and D.

      The "hallmark" patterns are shown in table 1 and are  
10 for LL: distinctive 29/33 KDa doublet, and for TT: distinctive 64/65 KDa doublet (often observed as single band) or a very pronounced smear in the 30-64 KDa region.

      To Blot A and B are applied: Lane 1: BCG crude extract, Lane 2: crude extract of a M. tuberculosis strain, Blot C:  
15 Lane 1: Molecular marker, Lane 2: not relevant, Lane 3: BCG crude extract, Lane 4: crude extract of an M. tuberculosis strain, Blot D: Lane 1: BCG crude extract, Lane 2: crude extract of an M. tuberculosis strain, Lane 3: Molecular marker.

20

Interpretation of banding patterns, wherein only the "hallmarks" are mentioned, is as follows:

Blot A/C: 29-33 KDa doublet in lane 1 and 2.

Blot B/D: 64/65 KDa doublet in lane 1 and 2.

25        Noticeable is the very intensive smear in the 30-64 KDa range on blot D.

      Finally, figure 2c is an example of different Western blotting patterns developed after incubation with representative sera of patients with Crohn's Disease. The  
30 "hallmark" patterns are shown in table 3 and are for Crohn's Disease a pronounced 45/48 KDa doublet.

      Applied to blot A is: BCG crude extract,  
blot B: crude extract of an M. tuberculosis strain,  
blot C: Mycobacterium avium crude extract,  
35 blot D: molecular marker.

Interpretation of banding patterns is as follows:

All lanes show a distinctive colouring of the 45/48 KDa doublet positivity, which indicates Crohn's Disease. The 45/48 KDa doublet reacts positively in 65% of all Crohn's patients.

#### EXAMPLE 2. Enzyme Immunoassay

##### 1. Preparation of antigens

The starting material (see EXAMPLE 1 under 1.) was, depending on the chosen M. tuberculosis strain, centrifuged at 70,000 x g to 120,000 x g at 4°C for 2 hours. The pellet was washed three times with PBS. Between the washing steps centrifuging took place at 70,000 x g to 120,000 x g at 4°C for 2 hours. The pellet was collected and resuspended in 10 ml PBS. The supernatant, SP-100, can also be used for immunoblots (example 1) and enzyme immunoassays (this example). The suspension was subsequently sonified for 2 minutes at 80 watts at 4°C. After the protein concentration was determined, quantities of 100 µl were frozen at a concentration of 1 mg/ml and stored at -80°C until time of use (this preparation is designated with the term KP-100).

30 mg of the starting material was then applied in the presence of loading mixture onto a preparatory 12% polyacrylamide gel of 0.5 cm thickness after 20 minutes of incubation at 65°C. Electrophoresis was carried out for 30 minutes at 150 V (stacking gel) and for 6 hours at 100 V (running gel). The electrophoresis was stopped after the blue colorant band had disappeared from the gel. The gel was then cut into horizontal strips of 2 mm thickness which in turn were divided into pieces of 1 cm length. The gel pieces were each eluted overnight at 4°C in a tube with 5 ml sterile distilled water. Thorough mixing thereafter took place and the remaining gel pieces were centrifuged to the bottom.

The elution was checked using a 12% polyacrylamide analytical gel of 1.5 mm thickness. The gel was cast with a comb. After 20 minutes incubation at 65°C 40 µl of each tube with gel pieces was placed in the slots in the presence of

10  $\mu$ l 5x loading mixture. The electrophoresis was carried out for 30 minutes at 150 V in the "stacking gel" and for 6 hours at 100 V in the "running gel". The electrophoresis was stopped and the gel made ready for preparation of a Western blot. The blotting procedure is described in EXAMPLE 1 under 5 2. Similar results were obtained using HPLC, FPLC and other routine separating procedures.

In order to establish which fractions contain the relevant antigens, strips of the blot were incubated with 10 sera of patients with lepromatous leprosy, tuberculous leprosy and Crohn's Disease. Shown herewith are respectively the 29/33 KDa antigens, the 64/65 KDa antigen and the 45/48 KDa antigens. The complex formation was visualized using anti-human IgG peroxidase conjugate and DAB. The desired 15 fractions were collected, combined and used to coat a microtiter plate (see below).

## 2. EIA.

Microtiter plates are coated (via standard techniques) 20 with either KP-100, SP-100, starting material, whole bacteria, 29/33 KDa, 64/65 KDa or 45/48 KDa antigens.

After coating the plates are blocked, in order to prevent an aspecific binding of serum components, with a 3% bovine serum albumin (BSA) solution. Plates are then dried 25 and stored at 4°C.

### 2.1. Tuberculosis EIA test (microtiter plates coated with KP-100)

Test sera are pipetted in a 1:100 dilution into the 30 coated wells of a microtiter plate. The reaction takes place for 1 hour at 37°C. Aspecific serum components and non-binding serum components are washed away in a washing step. A second incubation with a suitable dilution of an anti-human IgA peroxidase conjugate is carried out again for 1 35 hour at 37°C, and excess conjugate is then washed away.

Indication of human antibodies of the sub-type IgA binding specifically to KP-100 takes place by adding TMB (tetramethylbenzidine) to the wells.

Binding enzyme results in the occurrence of a blue colour which, after addition of a colouring stop solution, changes to yellow. This yellow colour has an absorption maximum of 450 nm.

- 5       The intensity of the resulting colour is proportional to the amount of bound KP-100-specific IgA.

The results are shown in the tables below.

In the described test patient and control sera are used from two different populations.

- 10   A = Endemic area (Africa, Ghana)

B = Non-endemic area (Europe, the Netherlands).

Each population is sub-divided into 4 sub-groups, namely:

Group 1 = culture confirmed TB patients

- 15   Group 2 = negative control group (normal healthy individuals)

Group 3 = suspected positives (TB contacts)

Group 4 = suspected negatives (no known data, but certainly no TB, possibly leprosy or other aspecific Mycobacteriosis).

- 20       The test is performed with two kits having different lot numbers and production dates.

Interpretation of the test results takes place on the basis of the so-called calibration line which is made up of control sera with a determined arbitrary unit definition

- 25   which corresponds to a known OD value (1 unit, 4 units and 8 units).

Each time a test is carried out the units are included in the assay. Found sample values can then be related to the unit definition.

- 30       A test serum can be considered positive when the result found in the test scores higher than 2.1 units.

A test serum can be considered negative when the result found scores lower than 1.2 units.

- 35       Test sera with unit values between 2.1 and 1.2 units fall into the set so-called reconfirmation zone. This means that in the first instance positivity or negativity for tuberculosis cannot be determined with this test.

Reconfirmation of these sera takes place using the described Western blot strips with which, after serum incubation on the basis of banding patterns and specific "hallmarks", an answer can be given to the question of whether the test serum is positive (bands present) or negative (bands absent).

**Table 3:**

Population A. Endemic range:

Group 1:

SERUM NO.	# UNITS	TEST SCORE	REMARKS
1	2.44	positive	culture positive
2	3.43	positive	culture positive
3	1.78	reconfirmation	culture positive
4	1.37	reconfirmation	culture positive
5	5.74	positive	culture positive
6	3.21	positive	culture positive
7	1.66	reconfirmation	culture positive
8	2.00	reconfirmation	culture positive
9	2.00	reconfirmation	culture positive

**Table 4:**

Population A. Group 2:

SERUM NO.	# UNITS	TEST SCORE	REMARKS
10	1.16	negative	healthy individual
11	0.86	negative	healthy individual
12	0.77	negative	healthy individual
13	0.64	negative	healthy individual
14	0.74	negative	healthy individual
15	0.79	negative	healthy individual

Table 5:

Population A. Group 3:

SERUM NO.	# UNITS	TEST SCORE	REMARKS
16	3.13	positive	sick individual
17	1.57	reconfirmation	sick individual
18	1.59	reconfirmation	sick individual
19	5.39	positive	sick individual
20	1.97	reconfirmation	sick individual
21	2.29	positive	sick individual
22	0.48	negative	sick individual

Table 6:

Population A. Group 4:

SERUM NO.	# UNITS	TEST SCORE	REMARKS
23	1.54	reconfirmation	normal control
24	1.76	reconfirmation	normal control
25	0.58	negative	culture negative
26	1.03	negative	culture negative
27	0.79	negative	culture negative
28	0.89	negative	culture negative



**Table 7:**

Population B. Non-Endemic range:

Group 1:

SERUM NO.	# UNITS	TEST SCORE	REMARKS
1	2.24	positive	culture positive
2	2.53	positive	culture positive
3	4.40	positive	culture positive
4	14.95	positive	culture positive
5	16.82	positive	culture positive
6	10.54	positive	culture positive
7	5.70	positive	culture positive
8	6.72	positive	culture positive
9	5.06	positive	culture positive

**Table 8:**

Population B.

Group 2:

SERUM NO.	# UNITS	TEST SCORE	REMARKS
10	1.04	negative	healthy individual
11	0.92	negative	healthy individual
12	0.85	negative	healthy individual
13	0.20	negative	healthy individual
14	0.42	negative	healthy individual
15	0.90	negative	healthy individual
16	0.35	negative	healthy individual
17	0.73	negative	healthy individual

**Table 9:**  
Population B.  
Group 3:

SERUM NO.	# UNITS	TEST SCORE	REMARKS
18	2.23	positive	sick individual
19	4.70	positive	sick individual
20	1.22	reconfirmation	sick individual
21	1.43	reconfirmation	sick individual
22	2.21	positive	sick individual with TB history
23	6.38	positive	sick individual
24	1.59	reconfirmation	sick individual

**Table 10:**  
Population B.  
Group 4:

SERUM NO.	# UNITS	TEST SCORE	REMARKS
25	0.20	negative	patient resistant to drug therapy

## CLAIMS

1. Method for identifying a Mycobacterium species responsible for a mycobacterial infection in human or animal, comprising the steps of:

5 a) selecting a suitable mycobacterial species and strain;

b) preparing at least one mycobacterial antigen, respectively antigen preparation;

c) binding the antigen, respectively the antigen preparation to a suitable carrier;

10 d) causing the bound antigen to react with antibodies from serum of an individual infected with a Mycobacterium species;

e) making visible antigen-antibody reactions for a suitable antibody (sub-)class; and

15 f) identifying the responsible Mycobacterium species on the basis of the reactions which are made visible.

2. Method as claimed in claim 1, characterized in that the antigen preparation is separated by electrophoresis prior to step c) and the carrier is a membrane to which the antigen is bound by means of electroblotting.

3. Method as claimed in claim 2, characterized in that the membrane is a nitrocellulose membrane.

4. Method as claimed in claim 1, characterized in that the Mycobacterium species responsible for the mycobacterial infection is identified on the basis of a reference pattern.

5. Method as claimed in any of the claims 1-4, characterized in that the antigen preparation is a total protein preparation of Mycobacterium.

6. Method as claimed in any of the claims 1-4, characterized in that the antigen preparation is a KP-100 or SP-100 fraction of the total protein preparation.

7. Method as claimed in claim 1, characterized in that the antigen is specific for one Mycobacterium species and the carrier is a microtiter plate.

8. Method as claimed in claim 7, characterized in that the specific antigen is chosen from the group consisting of

mycobacterial immuno-cross-reactive antigen components with a molecular weight of respectively 29/33 KDa, 45/48 KDa, 64 KDa and the antigen fractions KP-100 or SP-100 or combinations thereof.

5           9. Method as claimed in claim 7 or 8, **characterized in that** the antigen-antibody reactions are made visible using antibody-enzyme conjugates directed against antibodies of an isotype chosen from IgG, IgM, IgA.

10           10. Method as claimed in claim 9, **characterized in that** the enzyme is peroxidase.

15           11. Method as claimed in claim 1, **characterized in that** the carrier is a dip-stick with mycobacterial antigens binding thereto, that the antigen is brought into reaction with antibodies from serum for testing by dipping the stick in a serum monster for testing, and that the antigen-antibody reactions are made visible by subsequently dipping the stick in a solution with anti-antibody antibody-enzyme conjugate and a colour substrate for the relevant enzyme.

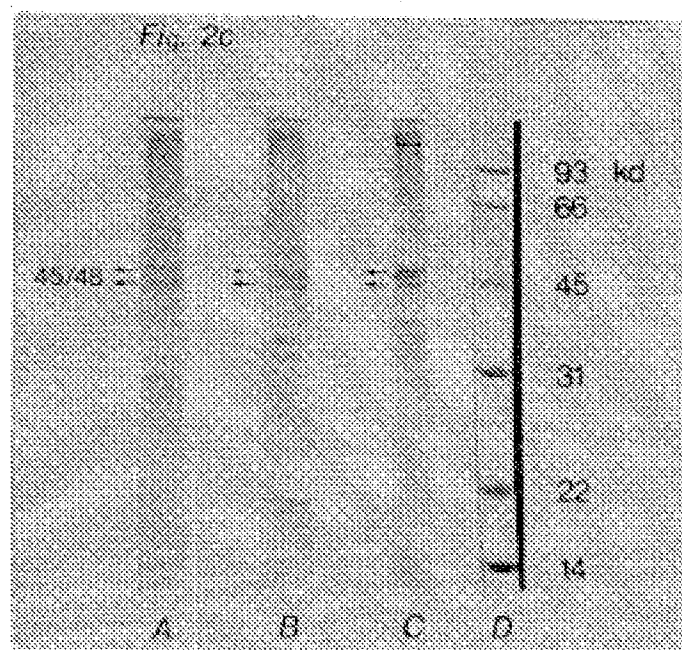
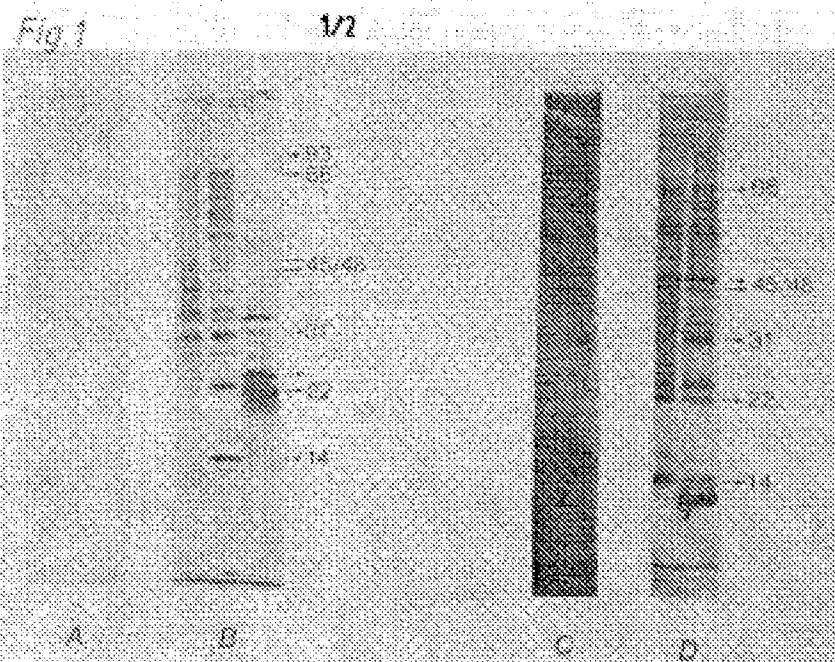
20           12. Method as claimed in claim 1, **characterized in that** the antigen is bound to the wells of a microtiter plate, that the antigen is brought into reaction with antibodies from the serum to be tested by placing in the wells a sample of the serum to be tested, that the non-binding antibody is removed, and that the antigen-antibody reaction is made visible by subsequently placing in the wells an anti-antibody antibody-enzyme conjugate and a colour substrate for the relevant enzyme.

30           13. Diagnostic kit comprising a dip-stick on which is arranged a carrier strip with mycobacterial antigens bound thereto, and means for visualizing antigen-antibody reactions occurring on the carrier after contact with the serum for testing.

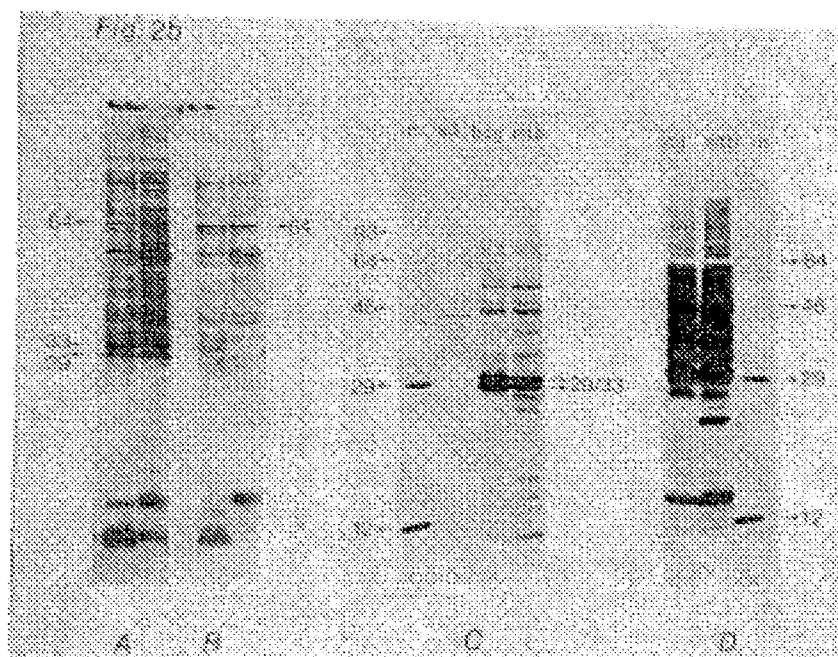
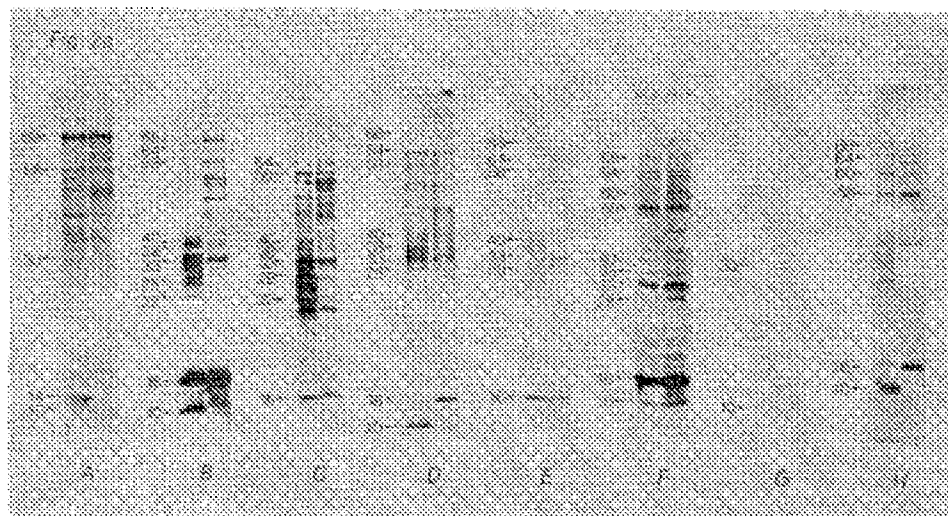
35           14. Diagnostic kit comprising a microtiter plate in the wells of which a specified antibody is arranged, and means for visualizing antigen-antibody reactions occurring in the wells after contact with the serum for testing.

          15. Diagnostic kit comprising an immunoblot with mycobacterial antigens separated by electrophoresis binding

thereto, and means for visualizing antigen-antibody reactions occurring on the immunoblot after contact with the serum for testing.



2/2



## INTERNATIONAL SEARCH REPORT

Date of Application No.

PCT/NL 93/00270

A. CLASSIFICATION OF SUBJECT MATTER  
 IPC 5 G01N33/569 G01N33/543

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
 IPC 5 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF CLINICAL MICROBIOLOGY vol. 26, no. 12, 1 December 1988, CHICAGO IL USA pages 2474 - 2479 F. VEGA-LOPEZ ET AL. 'Recognition of Mycobacterial antigens by sera from patients with leprosy.' see figures 1-5	1-6
Y	see the whole document --- -/--	7,9-15

☒ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

\* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another claim or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"Z" document member of the same patent family

Date of the actual completion of the international search

28 March 1994

Date of mailing of the international search report

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## INTERNATIONAL SEARCH REPORT

Int. Application No.

PCT/NL 93/00270

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>INTERNATIONAL JOURNAL OF LEPROSY AND OTHER MYCOBACTERIAL DISEASES vol. 60, no. 2 , 1 June 1992 , WASHINGTON DC USA pages 201 - 207 P.W. ROCHE ET AL. 'Antibody response to the 18-kDa protein of Mycobacterium leprae in leprosy and tuberculosis patients.' see table 2</p>	7,9-15
X	<p>INFECTION AND IMMUNITY vol. 59, no. 2 , 1 February 1991 , CHICAGO IL USA pages 665 - 670 D.V. HAVLIR ET AL. 'Human immune response to Mycobacterium tuberculosis antigens.' see page 665, line 6 - line 7</p>	8
A	<p>SCANDINAVIAN JOURNAL OF IMMUNOLOGY vol. 33, no. 6 , 1 June 1991 , OXFORD UK pages 763 - 775 A. RAMBUKKANA ET AL. 'Subcellular distribution of monoclonal antibody defined epitopes on immunodominant Mycobacterium tuberculosis proteins in the 30-kDa region: identification and localization of 29/33-kDa doublet proteins on Mycobacterial cell wall.' see the whole document</p>	1-15